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14. ABSTRACT

On the basis of previous studies in which we analyze AR phosphorylation in vivo, we propose that AR phosphorylation at serines 213 and 650 regulate differential target gene expression and recruitment to gene promoters via altered interaction with other cellular transcription factors. To test this hypothesis we have conducted yeast two-hybrid analysis with the N-terminus of wild type AR as well as AR S213A and AR S213E variants. Our preliminary analysis indicates that the screen is preferentially isolating proteins with a known role in gene transcription and we are currently assessing the phosphorylation-dependence of the putative AR interacting proteins.

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INTRODUCTION:

The androgen receptor regulates prostate cell growth and differentiation and plays a critical role in prostate cancer progression. Like other steroid hormone receptors, AR is a phospho-protein and phosphorylation is believed to regulate AR function. To provide insight into the regulation and function of AR phosphorylation, we generated novel antiserum that specifically recognizes AR phosphorylated on key serine residues. Utilization of these antibodies indicates that AR phosphorylation is tightly regulated in urogenital developmental and in differentiated adult prostate. Thus, the development of AR phosphorylation site-specific antibodies along with AR mutant molecules provides a unique opportunity to study the regulation of AR phosphorylation by cellular kinases as well as the impact of phosphorylation on AR function.

Using our novel anti-serum that specifically recognizes AR phospho-serine 213 (P-S213), a putative site of Akt phosphorylation, we demonstrated rapid phosphorylation at S213 in response to agonists R1881 and DHT, but not in response to antagonists bicalutamide or flutamide. By immunohistochemistry, the AR-P-S213 antigen was detected in prostate epithelial but not stromal cells despite the fact that an antibody recognizing both phosphorylated and non-phosphorylated forms of AR demonstrates that AR is present in both cell types as expected. In fetal tissue, the AR-P-S213 antigen was present in epithelial cells of the urogenital sinus when endogenous androgen levels are high, but absent at a later stage of development when endogenous androgen levels are low. Immunoreactivity is evident in differentiated cells lining the lumen of the urogenital sinus, but not in rapidly dividing, Ki67 positive cells within the developing prostate or stromal tissue, suggesting that site-specific phosphorylation of AR S213 by cellular kinases occurs in a non-proliferating cellular milieu. The exquisite cell type specificity of AR S213 phosphorylation suggests that phosphorylation is tightly regulated by cellular kinases and may function in AR-mediated transcription in a specified cellular context.

Examination of phosphorylation of AR at serine 650 (S650) was also conducted. Interestingly, phosphorylation of S650 is enhanced by treatment with forskolin (FSK), Epidermal Growth Factor (EGF) and phorbol-12-myristate-13-acetate (PMA)[Gioeli, D., J. Biol. Chem., 2002] suggesting that AR phosphorylation may be intricately linked to signal transduction processes regulating tumor promotion and cell growth. Consistent with the idea that multiple cell signaling pathways contribute to phosphorylation at AR S650, we find enhanced phosphorylation of AR S650 following R1881 treatment using antibody against AR phospho-serine 650. Characterization of signaling pathways that contribute to phosphorylation of AR S650 is in progress.

BODY

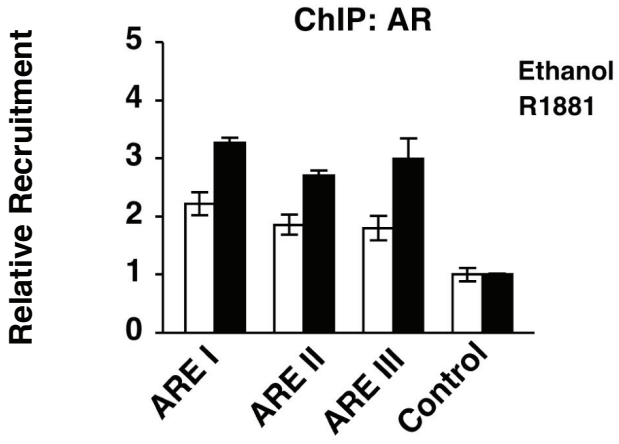
The original grant outlined two tasks in the statement of work. Each task is listed below followed by a description of the research progress relevant to the task.

Task 1. Determine the effect of phosphorylation on AR-mediated gene transcription. Transcription of AR target genes will be compared in HPr-1 prostate cell lines stably expressing wild type AR versus phosphorylation site mutants.

We have decided to expand this aim a little to include a tumorigenic cell line (PC3) and well as an immortalized, non-transformed cell line (HPr-1) as originally planned. PC3 cells have been transfected with wild type and mutant AR and we are in the process of analyzing individual clones. HPr-1 cells, on the other hand, turn out to be difficult to transfect so we have changed strategies and plan to infect them with retrovirus. We have cloned the wild type receptor into the retrovirus vector and are working on obtaining the mutants in the same vector. The retrovirus will enable us to select GFP positive AR containing pools. The cell selection will be done through the cell sorting facility of the NYU Cancer Institute. As soon as the cell lines are constructed we will isolate RNA and conduct gene array to identify target genes that are differentially affected by AR phosphorylation.

Task 2. Examine the effect of AR phosphorylation on recruitment of coactivators and corepressors to the androgen-regulated PSA promoter.

In preparation for the completion of this aim, we have shown that we can detect AR recruitment to the PSA promoter via chromatin immunoprecipitation assays. We are now ready to conduct experiments with the phospho-antibodies.



Task 3. Characterize proteins that interact with AR S213 and S650 in a phosphorylation dependent manner via yeast two-hybrid analysis.

<u>This aim is the most labor-intensive aim in the grant and therefore we have dedicated the most time to initiating and conducting this aim</u>.

We have proposed that phosphorylated S213 and S650 act as docking sites to recruit coactivators or corepressors to AR. To test this hypothesis, we have conducted yeast two-hybrid experiments to begin to characterize proteins that interact with phosphorylated AR.

Because the AR N-terminal activation domain is transcriptionally active in yeast and therefore cannot be used as "bait" in a conventional two-hybrid screen we have utilized a "reverse two-hybrid" system in which a library is created that fuses cDNAs to a DNA binding domain, rather than to an activation domain. To identify proteins that bind to AR S213 or S650, we will utilize

serine to glutamic acid mutations that mimic the phosphorylated form of the receptor (AR S213E and AR S650E).

Human AR_{1-488} S213E was constructed, sub-cloned into a galactose-inducible expression vector (pJG 4-5), and expressed as a hybrid protein fused to an acidic B42 transcriptional activation domain ("the bait"). A random- primed normal prostate cell cDNA library cloned into a yeast expression vector (pEG 202) was linked to the Lex A DNA binding domain ("the prey") and represents ~$1x10^6$ cDNAs. The auxotrophic yeast strain EGY 188 (*trp1 his3 ura3 leu2*), with a chromosomally integrated Lex A-responsive LEU2 reporter gene, was transformed with 1) the AR_{1-488} S213E bait, the 2) library prey, and 3) a β-galactosidase (β-gal) reporter gene linked to a single Lex A operator. Library proteins that interact with AR_{1-488} (bait-prey interactions) serve to reconstitute transcription and activate LEU2 and β-gal reporter gene expression. Expression of the Lex operator-linked LEU2 reporter allows for auxotrophic EGY 188 cells to grow in the absence of leucine, while β-galactosidase cleaves the chromogenic substrate X-gal, causing the colonies to appear blue. Glucose represses the galactose-inducible promoter, inhibiting production of the AR_{1-488} bait protein. The library was transformed into the strain containing AR_{1-488} and selected for colonies that grow and are blue on galactose, leucine-deficient X-gal plates. We have obtained hundreds of colonies that are blue on galactose X-gal plates, but white on glucose X-gal plates, and are currently characterizing these further. Approximately 45 clones have been isolated from yeast, transformed into bacteria and sequenced. Most of these contain identifiable proteins in BLAST searches. Clones that consisted of vector only or of short peptide sequences were discarded. All interesting clones have been re-transformed into yeast to verify the original result. Of the clones with which we have preceded the farthest, 8 out of 12 of the corresponding proteins have a documented role in transcription giving credence to the quality of the screen.

Proteins that interact with the AR_{1-488} S213E are currently being subjected to an additional screen that will identify proteins that specifically associate with the AR in a phosphorylation site-dependent manner. By comparing the β-gal expression induced by the interaction of library proteins with AR_{1-488} S213E *vs.* AR_{1-488} S213A we can identify proteins that associate with AR in a manner dependent on phosphorylation. Because the protein concentrations are so high in two-hybrid assay, we have followed up on differences in visualized on the yeast plates using liquid β-gal assays. While we have only preformed these experiments on a small number of proteins so far, at least one clone shows enhanced affinity for AR S213E suggesting that it may interact with the AR in a phosphorylation-dependent manner. Another clone interacts preferentially with AR S213A, suggesting that it might not interact with the AR when it is phosphorylated at S213.

KEY RESEARCH ACCOMPLISHMENTS

- A reverse yeast two-hybrid screen has identified multiple proteins that interact with the AR phosphorylation-site mimetic S213E

- The identity of a dozen proteins has been confirmed so far and the majority play a documented role in gene transcription

REPORTABLE OUTCOMES

Abstracts and Publications, 2005-2006

Taneja, S.S., Ha, S., Swenson, N.K., Huang, H.Y., Lee, P., Melamed, J., Shapiro, E., Garabedian, M.J. and **Logan**, S.K. Cell specific regulation of androgen receptor phosphorylation *in vivo*. J Biol Chem. 2005 Oct 6; [Epub ahead of print]

Ha, Susan, Swenson, N.K., Huang, H-Y, Shapiro, E., Garabedian, M.J., Taneja, S.S. and **Logan**, S.K. Cell specific regulation of androgen receptor phosphorylation *in vivo*. Keystone Symposia, Hormonal Regulation of Tumorigenesis. Monterey, California, 2/20/2005, abstract

Susan Ha, Rachel Ruoff, Hong Ying Huang, Ellen Shapiro, Michael J. Garabedian, Samir S. Taneja and Susan K. Logan Cell specific regulation of androgen receptor phosphorylation *in vivo*. Keystone Meeting, Steroid Sisters, 3/18/2006, abstract

Susan Ha, Rachel Ruoff, Hong Ying Huang, Ellen Shapiro, Michael J. Garabedian, Samir S. Taneja and Susan K. Logan. Regulation of androgen receptor phosphorylation in prostate cancer. Cold Spring Harbor Meeting. 11/1/2006, abstract

Research training during the period of grant support (under my immediate supervision):

Raluca Pancratof	Sackler rotation student	1/15-06-present
Andrew Hanover	NYU undergraduate	1/30/06-5/29/06
Derick Mitchell	postdoctoral fellow	9/1/06-present

In advisory function (thesis committee):

Jason Lieberthal	M.D./Ph.D. candidate-	Naoko Tanese, advisor
Jerome Nwachukwu	Ph.D. candidate-	Michael Garabedian, advisor
Diah Douglas	MD/honors thesis, 2006	Iman Osman/Peng Lee, advisors
Vladimir Liarski	MD/honors thesis, 2006	Leslie Gold, advisor
Jon Baghdadi	MD/honors thesis-	Susan Logan, advisor
Pricilla Maldonado	Sackler student-	Jim Borowiec, advisor
Raluca Pancratof	PhD candidate-	Susan Logan/Ram DasGupta, advisors
Andrew Goldsweig	MD/PhD candidate-	Harry Ostrer, advisor

CONCLUSION:

We propose that AR phosphorylation at serines 213 and 650 regulate differential target gene expression and recruitment to gene promoters via altered interaction with other cellular transcription factors. To test this hypothesis we have conducted yeast two-hybrid analysis with the N-terminus of wild type AR as well as AR S213A and AR S213E variants. Our preliminary analysis indicates that the screen is preferentially isolating proteins with a known role in gene transcription and we are currently assessing the phosphorylation-dependence of the putative AR interacting proteins.